Orientation of melittin in phospholipid bilayers

A polarized attenuated total reflection infrared study

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ABSTRACT The helical order parameter of the 26-residue amphiphilic bee venom peptide melittin was measured by polarized attenuated total reflection infrared spectroscopy (ATR-IR) in dry phospholipid multibilayers (MBLs) and when bound to single supported planar bilayers (SPBs) under D_2O. Melittin adopted an α-helical conformation in MBLs of dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), a 4:1 mixture of POPC and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), and when bound to SPBs of POPC:POPG (4:1). The order parameter of the α-helix in the bilayers depended mainly on the type of membrane preparation, and only little on the phospholipid composition of the bilayers. On hydrated SPBs, the helical order parameter was negative, indicating that the α-helix long axis of melittin was preferentially oriented parallel to the plane of the supported membrane. However, in dry MBLs, the helical order parameter was positive, indicating that the α-helix of melittin was preferentially oriented parallel to the phospholipid fatty acyl chains. It is concluded that the orientation of melittin in membranes depends on the degree of hydration of the model membranes rather than on the technique which is used for its determination. ATR-IR spectroscopy of polypeptides in or associated with supported planar membranes in D_2O may become a useful tool for the determination of their orientation in and on membranes.

INTRODUCTION

Melittin is one of the best studied membrane-interactive polypeptides (see Dempsey, 1990, for a recent review). The 26-residue peptide (Habermann and Jentsch, 1967) is the major component of the venom of the honey bee *Apis mellifera.* Melittin binds spontaneously to biological and phospholipid model membranes and causes a significant membrane disruption and hemolysis in erythrocytes (Sessa et al., 1969; Habermann, 1972). At high concentrations, melittin acts as a detergent and entirely dissolves membranes. At lower concentrations, melittin induces voltage-gated channels in black lipid membranes (Tosteson and Tosteson, 1981; Kempf et al., 1982; Tosteson et al., 1987). Other actions of melittin on membranes include its capabilities to induce membrane fusion (Morgan et al., 1983; Murata et al., 1987; Bradick et al., 1989), nonbilayer lipid structures (Batenburg et al., 1987), lipid phase separations (Lafleur et al., 1982) and the aggregation of integral membrane proteins in erythrocytes (Clague and Cherry, 1988).

In dilute aqueous solution, melittin is monomeric and does not assume any ordered secondary structure as determined by CD or ^{1}H-NMR spectroscopies (Talbot et al., 1979; Lauterwein et al., 1980). However, when bound to membranes or detergent micelles, melittin adopts a highly α-helical conformation (Dawson et al., 1978; Knoppel et al., 1979; Brown et al., 1982; Vogel and Jahnig, 1986, Vogel, 1987). The crystal structure of α-helical melittin has been solved by x-ray crystallography to a 2-Å resolution (Terwilliger et al., 1982). This structure is highly amphiphilic, with most hydrophobic residues on one side and most hydrophilic residues on the opposite side of the helix long axis. Four of the six positively charged residues are located near the C-terminus. This charge distribution introduces further asymmetry into the structure of α-helical melittin.

The orientation of the helix in lipid bilayers is controversial. Vogel et al. (1983) used polarized transmission IR spectroscopy to determine the orientational order parameter of melittin in dry multibilayers and, more recently, Brauner et al. (1987) reinvestigated this problem by using polarized ATR-IR spectroscopy. It was concluded from these studies that the α-helical portion of melittin was preferentially oriented parallel to the lipid fatty acyl chains. CD studies on partially hydrated (97% relative humidity) oriented multibilayers appeared to confirm these results (Vogel, 1987). In contrast, accessibility measurements of spin-labeled melittin by chromium oxalate (Altenbach and Hubbell, 1988; Altenbach et al., 1989) and ^{13}C-NMR measurements in the presence of aqueous shift reagents (Stanislawski and

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Abbreviations used in this paper: ATR, attenuated total reflection; CD, circular dichrosim; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; IR, infrared; MBL, multibilayer; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylglycerol; SPB, supported planar bilayer; TLC, thin layer chromatography.

0006-3495/91/10/922/09 $2.00

Ruterjans, 1987) indicated a location of melittin on the membrane surface with only the hydrophobic residues buried in the lipid bilayer. These magnetic resonance experiments were performed with sonicated phospholipid vesicles. It is unclear, therefore, whether the discrepancy of the different reports on the orientation of melittin in membranes originates from the technique or from the type of model membrane preparation which was used for its determination. To resolve this question, we have used a single technique (ATR-IR spectroscopy) to determine the order parameter of melittin bound to two different types of model membranes, namely, fully hydrated single supported planar bilayers and dry multibilayers. We find a *negative* helical order parameter (consistent with an orientation parallel to the plane of the membrane) in SPBs under D_2O, and a *positive* helical order parameter (consistent with an orientation parallel to the fatty acyl chains) in dry MBLs.

MATERIALS AND METHODS

Materials

DPPC, POPC, and POPG were from Avanti Polar Lipids (Alabaster, AL). They were shown to be pure by silica gel thin layer chromatography and were used without further purification. Melittin (Serva Biochemicals, Heidelberg, Germany) was dissolved in methanol. All insoluble material was removed by centrifugation (15,000 *g*). This treatment eliminated residual phospholipase activities as assayed by incubating test samples with small unilamellar phospholipid vesicles of lipid and analysing the reaction products by TLC after several hours or days. Some melittin was a kind gift of Dr. H. Vogel. This sample was purified by gel filtration on sephadex G75 as described (Vogel and Jahnig, 1986). Both melittin preparations gave identical IR results. 99.8% isotopically pure D_2O was from Dr. Glaser AG (Basel, Switzerland).

Preparation of model membranes

Single planar bilayers were prepared by a combined Langmuir-Blodgett/vesicle-fusion technique, similar to that described by Fringeli (1989). The Ge ATR-plate was cleaned by wiping the surface with a tissue soaked in chloroform/ethanol (1:4) and, immediately before use, by argon plasma cleaning (Harrick, Ossining, NY). A single monolayer of POPC was withdrawn from a Fromherz circular monolayer trough (Meyer, Gottingen, Germany) at a surface pressure of 32 mN/m as described (Tamm, 1988). The plate was then assembled with a liquid cell which was home made from polyacetate. The cell was filled with 0.7 ml of a 1-mM solution of sonicated small unilamellar vesicles of POPC:POPG (4:1) in 10 mM Na-phosphate buffer, pH 7.3, containing 0.15 M NaCl. Some of these vesicles fused spontaneously with the supported monolayer to give a single planar bilayer (Fringeli, 1989; E. Kalb, S. Frey and L. Tamm, unpublished results). After 60 min of incubation, the cell was flushed with 10 vol of H_2O and 5 vol of D_2O to remove excess vesicles and to exchange H_2O for D_2O. Precautions were taken to avoid any contact of the bilayer with air.

Multibilayers of phospholipids or mixtures of phospholipids and melittin were prepared as described (Fringeli and Gunthard, 1981). Typically, 10 μl of a 5-mM solution of lipid or lipid/peptide in chloroform/methanol (4:1) was deposited on the germanium ATR plate (52 × 20 × 1 mm, 45°; Dr. Karl Korth Monokristalle, Kiel, Germany) and spread with a thin glass rod. The rod was moved slowly back and forth along the surface until the solvent had evaporated. Some multibilayers of pure lipids were prepared from chloroform solutions and some multibilayers of lipid/melittin from solutions of hexane:ethanol (2:1) instead of chloroform:methanol (4:1). These changes of the spreading solvent did not significantly change the ATR-IR spectra. Some samples were "reworked" with chloroform as described (Brauner et al., 1987), to produce multibilayers which were uniformly irridescent (Fringeli and Gunthard, 1981).

Infrared spectroscopy

Infrared spectra were recorded on a Perkin-Elmer 1800 Fourier-transform infrared spectrometer. The resolution was 2 cm^{-1} and a triangular apodization was used. For each polarization, 400 scans were collected with multibilayers and 4,000 scans with single planar bilayers, respectively. A goldwire grid polarisor (Perkin-Elmer, Beaconsfield, England) was used. Spectra of the plain germanium ATR-plate were recorded in each polarization and subtracted from the respective multibilayer spectra. For the single supported planar bilayers, difference spectra were obtained as follows: 4,000 scans were first accumulated in each polarization and in the absence of peptide. A 1–1.2 ml solution of 2 μM melittin in D_2O was then flown into the ATR-cell assembly and incubated for 1 h. Polarized spectra were recorded and the respective pure lipid spectra in each polarization were subtracted to yield the difference spectra of the peptide vibrations. The lipid vibrations were evaluated by calculating difference spectra between the sample, in the presence or absence of melittin, and the spectra of the plain germanium plate in D_2O. The vibrational bands of interest were sufficiently separated and it was not necessary to use band deconvolution routines to evaluate their absorption intensities.

Quantitative analysis of the polarized ATR-IR spectra

Fig. 1 depicts the setup for multiple reflection ATR-IR spectroscopy on SPBs and defines the coordinates and polarization vectors in a commonly used notation (Fringeli and Gunthard, 1981). The dichroic ratio is defined as the ratio of the absorption polarized parallel to the plane of incidence to that polarized perpendicular:

$$R^{ATR} = \frac{A_\parallel}{A_\perp} = \frac{E_x^2 k_x + E_z^2 k_z}{E_y^2 k_y}. \quad (1)$$

For thin films of refractive index n_2 on a substrate of refraction index n_1 and immersed into a bulk phase of refractive index n_3, the components of the electric field amplitudes in the film are (Harrick, 1979; Fringeli and Gunthard, 1981):

$$E_x = \frac{2\cos\gamma\sqrt{\sin^2\gamma - n_{31}^2}}{\sqrt{1-n_{31}^2}\cdot\sqrt{(1+n_{31}^2)\sin^2\gamma - n_{31}^2}}$$

$$E_y = \frac{2\cos\gamma}{\sqrt{2-n_{31}^2}}$$

$$E_z = \frac{2\cos\gamma\cdot n_{32}^2\cdot\sin\gamma}{\sqrt{1-n_{31}^2}\cdot\sqrt{(1+n_{31}^2)\sin^2\gamma - n_{31}^2}}, \quad (2)$$

where $n_{31} = n_3/n_1$, $n_{32} = n_3/n_2$ and γ is the angle of incidence of the IR beam at the germanium-solution interface. k_x, k_y, and k_z in Eq. 1 are the components of the integrated absorption coefficient in the fixed

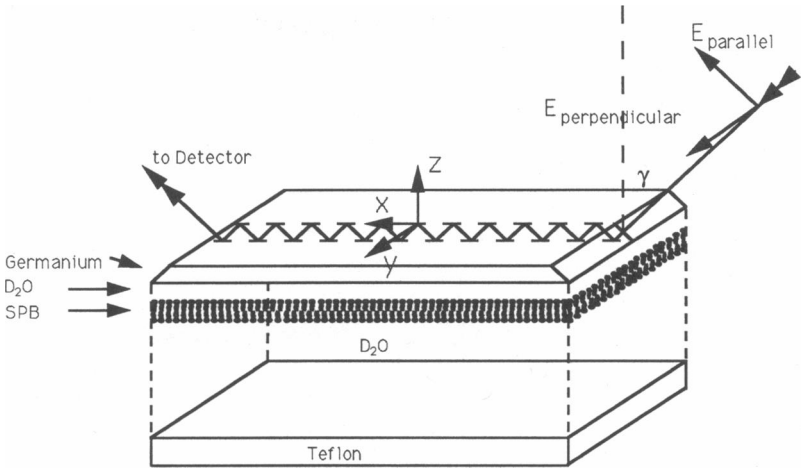

FIGURE 1 Schematic ATR setup for polarized infrared spectroscopy on supported single planar bilayers under D_2O. $E_{parallel}$ and $E_{perpendicular}$ define the electric field vectors of the incident polarized light. For simplicity, only the bilayer on the lower surface of the ATR plate is shown.

laboratory coordinate system. For an axially symmetric molecule whose director is oriented away from z by an angle θ and whose spectroscopically detected transition moment is oriented at an angle α from the director, these coefficients are (Fraser and MacRae, 1973):

$$k_x = k_y = K[(S\sin^2\alpha)/2 + (1 - S)/3]$$
$$k_z = K[S\cos^2\alpha + (1 - S)/3], \quad (3)$$

where K is a constant and the order parameter, $S = (3\langle\cos^2\theta\rangle - 1)/2$, is a function of the time- and space-averaged fluctuations of the molecular director around z.

Three vibrational modes are analyzed to determine the lipid order parameters, S_L; namely, the symmetric and antisymmetric CH_2 stretching modes and the C = O stretching mode. The transition moments of these three vibrations are oriented at right angles to the molecular director, i.e., $\alpha = 90°$ (Fringeli and Gunthard, 1981). Therefore,

$$S_L = \frac{2(E_x^2 - R^{ATR}E_y^2 + E_z^2)}{E_x^2 - R^{ATR}E_y^2 - 2E_z^2}. \quad (4)$$

The amide I band is used to determine the order parameter of the α-helix of the polypeptide in the membrane. The transition moment of this band is oriented at an angle α of 39–40° from the α-helix long axis (Tsuboi, 1962; Bradbury et al., 1962), although lower values have also been reported (29–34°, Miyazawa and Blout, 1961; 24–28°, Rothschild and Clark, 1979). The resulting order parameter for the helical part of a polypeptide with a fraction of residues in an α-helical conformation, f_H, is

$$S_H = \frac{E_x^2 - R^{ATR}E_y^2 + E_z^2}{f_H\left(\frac{3\cos^2\alpha - 1}{2}\right)(E_x^2 - R^{ATR}E_y^2 - 2E_z^2)}. \quad (5)$$

To evaluate Eqs. 4 and 5, a wavelength-independent refractive index $n_1 = 4.0$ was used for the germanium ATR-plate, and the wavelength-dependent refractive indices of the thin film (n_2) and the bulk media (n_3; for D_2O and air, respectively) were taken from Fringeli et al. (1989) and are listed in Table 1. Also listed in Table 1 are the derived numerical values for E_x^2, E_y^2, E_z^2, and $R^{iso} = (E_x^2 + E_z^2)/E_y^2$. R^{iso} is the

TABLE 1 **Optical parameters used for quantitative analysis of the ATR-IR spectra**

$\nu(cm^{-1})$	Absorption band	n_2*	n_3‡	E_x^2	E_y^2	E_z^2	R^{iso}§
Supported single planar bilayers (SPBs):							
2924	CH_2 antisymmetric stretch	1.50	1.20	1.98	2.20	0.989	1.35
2853	CH_2 symmetric stretch	1.50	1.20	1.98	2.20	0.989	1.35
1735	C = O stretch	1.41	1.32	1.97	2.24	1.93	1.74
1645	Amide I (deuterated)	1.40	1.32	1.97	2.24	1.99	1.76
Multibilayers (MBLs):							
2920	CH_2 antisymmetric stretch	1.50	1.00	1.99	2.13	0.449	1.14
2850	CH_2 symmetric stretch	1.50	1.00	1.99	2.13	0.449	1.14
1735	C = O stretch	1.41	1.00	1.99	2.13	0.576	1.20
1655	Amide I	1.40	1.00	1.99	2.13	0.592	1.21

*Refractive index of the thin film.
‡Refractive index of the bulk medium.
§Dichroic ratio expected for isotropic distribution ($S = 0$). $R^{iso} = (E_x^2+E_z^2)/E_y^2$.

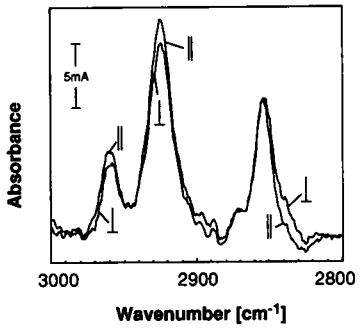

FIGURE 2 Parallel and perpendicular polarized ATR-IR difference spectra (germanium/SPB/D_2O-germanium/D_2O) of the CH_3 and CH_2 stretch region of a single planar bilayer of POPC/POPC:POPG (4:1).

ATR dichroic ratio expected for isotropic distribution ($S = 0$). The order parameters, S_L and S_H, were calculated from Eqs. 4 and 5, respectively, with the appropriate parameters of Table 1. Because the relevant bands of the experimental difference spectra are well isolated, peak heights rather than integrated peak areas were normally used to measure $R^{ATR} = A_\parallel/A_\perp$. Within experimental error, the height and area measurements gave identical results.

RESULTS AND DISCUSSION

Polarized attenuated total reflection infrared spectroscopy is a powerful tool to determine the order parameter of helical polypeptides in membranes. Because previous attempts to measure the orientation of melittin in membranes yielded conflicting results which depended on the physical method and type of model membrane which were used for its determination, we performed ATR-IR measurements on melittin which was bound to fully hydrated single supported planar bilayers on germanium substrates. These results were then compared with measurements of the order parameter of melittin in a more conventional membrane preparation, namely coplanar multibilayers prepared by solvent evaporation. We first describe and discuss the results which were obtained with the SPBs.

Asymmetric single supported planar bilayers were prepared on the germanium ATR-plates with a first leaflet of the SPB (exposed toward the substrate) of POPC and a second leaflet (exposed to the large aqueous compartment) of POPC/POPG (4:1). Polarized ATR-IR spectra were first recorded with SPBs under D_2O and in the absence of melittin. Difference spectra (germanium/SPB/D_2O − germanium/D_2O) were calculated for the whole range between 4,000 cm^{-1} and 800 cm^{-1}. The portions between 3,000 cm^{-1} and 2,800 cm^{-1} are depicted in Fig. 2 for parallel and perpendicular polarized light, respectively. Three bands can clearly be distinguished in this region: the antisymmetric CH_3 stretching mode at 2,960 cm^{-1}, the antisymmetric CH_2 stretching mode at 2,924 cm^{-1}, and the symmetric CH_2 stretching mode at 2,853 cm^{-1} (Bellamy, 1975). The shoulder at 2,870 cm^{-1} originates from the symmetric CH_3 stretching mode (Bellamy, 1975). The positions of these peaks indicate that the SPB is in a fluid phase (Cameron et al., 1980). The order of the fatty acyl chains in the SPB was assessed by measuring the ATR-dichroic ratios of both CH_2 stretching modes and the carbonyl stretching mode at 1,735 cm^{-1} which was also well resolved in these difference ATR-IR spectra (not shown). The numerical values of R^{ATR} for these three modes are listed in Table 2. Since the optical parameters which give rise to a particular value of R^{ATR} vary with the wavenumbers and (more significantly) with the refractive indices of the media in which the spectra are recorded (see Table 1), R^{ATR} values which are obtained under different conditions cannot be compared directly. In contrast, derived order parameters are intrinsic physical properties of the studied molecules in

TABLE 2 Dichroic ratios and derived order parameters of three characteristic lipid vibrations measured on supported single planar bilayer preparations

			2,924 cm^{-1}‡		2,853 cm^{-1}§		1,735 cm^{-1}‖	
Sample	Bulk solution	n*	R^{ATR}	S_L	R^{ATR}	S_L	R^{ATR}	S_L
Lipid bilayer	D_2O	7	1.14 ± 0.21	$0.37^{+0.54}_{-0.37}$	1.07 ± 0.18	$0.53^{+0.51}_{-0.37}$	1.32 ± 0.38	$0.39^{+0.51}_{-0.36}$
+ Melittin	D_2O	7	1.14 ± 0.10	$0.37^{+0.23}_{-0.19}$	1.02 ± 0.16	$0.65^{+0.49}_{-0.36}$	1.40 ± 0.33	$0.30^{+0.40}_{-0.29}$
+ Melittin	D_2O-buffer	3	1.08(±0.16)	$0.50^{(+0.44)}_{(-0.32)}$	1.02(±0.23)	$0.65^{(+0.77)}_{(-0.49)}$	1.41(±0.48)	$0.29^{(+0.62)}_{(-0.40)}$

*Number of measurements.
‡Antisymmetric CH_2 stretch.
§Symmetric CH_2 stretch.
‖Carbonyl stretch.

their environment and should be independent of the spectroscopic technique which was used to determine them. Therefore, the order parameters, S_L, were calculated for each of the three vibrational modes according to Eq. 4 (Table 2). The average order parameter of the asymmetric and symmetric CH_2 stretching vibrations is 0.45 ± 0.11. This value is in reasonable agreement with the molecular order parameters, S_{mol}, which are obtained in liquid-crystalline phospholipid bilayers by ^{2}H-NMR (Seelig and Seelig, 1980). Of course, unlike ^{2}H-NMR spectroscopy, IR spectroscopy cannot distinguish between order parameters for the individual methylene segments in a fatty acyl chain. Therefore, our values of S_L should be considered as average quantities which are used here only to establish that the lipids in the SPB are probably ordered in a similar fashion as in multilamellar coarse liposomes. Furthermore, and in contrast to S_{mol} from ^{2}H-NMR, the order parameters, S_L, are obtained from samples which are oriented on the germanium plate. Because the unoriented order parameter is related to the oriented order parameter by $S_{mol} = S_L \cdot (3\cos^2\theta'/2)$ (with θ' being the average orientation of the lipid director on the germanium plate) and because similar (or slightly larger) values are obtained for S_L as for S_{mol}, we conclude that the average orientation of the phospholipids in the SPB is perpendicular to the plane of the supporting germanium plate. Together with the order parameters derived from the carbonyl vibrations (Table 2), these data provide good evidence for a normal liquid-crystalline structure of the phospholipids in SPBs. These results are fully consistent with previous experiments on SPBs which have established that both leaflets of the supported bilayer are fully hydrated and that long range lateral diffusion of fluorescent phospholipid analogues occurs in both leaflets of the bilayer (Tamm and McConnell, 1985; Tamm, 1988).

In a next step, 2.1 nmol of melittin in D_2O were added to the liquid compartment of the ATR cell (cf. Fig. 1). After the peptide had bound to the membrane and reached equilibrium (after ~60 min), polarized ATR-IR spectra were recorded with the same spectrometer settings as were used before on the plain SPB. To assess the effect of melittin on the general lipid ordering, the same type of difference spectra were calculated as before (germanium/SPB[+ melittin]/D_2O − germanium/D_2O). The R^{ATR} values of the three lipid modes and the corresponding order parameters are listed in Table 2. The lipid order parameters were not significantly changed when melittin was bound to SPBs of POPC/POPC:POPG (4:1) (Table 2). Therefore, the integrity of the SPB appears to be largely preserved upon the binding of melittin.

To determine the orientation of melittin in supported planar bilayers, a different type of difference spectrum

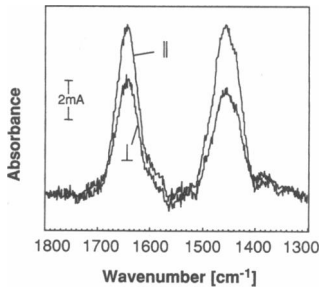

FIGURE 3 Parallel and perpendicular polarized ATR-IR difference spectra (germanium/SPB[+melittin]/D_2O-germanium/SPB[-melittin]/D_2O) of the amide I and amide II region of melittin bound to a single planar bilayer of POPC//POPC:POPG (4:1).

(germanium/SPB[+ melittin]/D_2O − germanium/SPB/[− melittin]/D_2O) was calculated. All predominant peaks in these difference spectra arise from the bound peptide, because the amount of unbound peptide in the evanescent field is negligible under the conditions of these experiments. The difference spectra were always calculated from parent spectra of the same SPB preparation, i.e., conditions which minimized experimental variations and instrumental drifts. The amide I and amide II regions of two such difference spectra which were obtained with parallel and perpendicular polarized light, respectively, are shown in Fig. 3. The amide I band was centered at 1,645 cm^{-1}, i.e., ~9 wavenumbers below the hydrogenated α-helical amide I band (see below). This isotope shift is larger than expected for α-helical proteins, but not quite as large as in deuterated α-helical polylysine (Susi et al., 1967). Six of the 26 residues of melittin are positively charged (*N*-terminus, 3 lysines, 2 arginines), and they or the low dielectric environment of the membrane may cause the observed amide I band shift. The amide II vibration was even further red-shifted to 1,460 cm^{-1} in D_2O as compared to ~1,550 cm^{-1} in H_2O. However, the observed peak at 1,460 cm^{-1} probably represents a superposition of the amide II vibration and the HOD bending mode which is also centered at ~1,460 cm^{-1} (Sadtler, 1984).[1] Therefore, this band was not used for further evaluation. No intensity was found around 1,550 cm^{-1} in the spectra of Fig. 3, indicating that all amide protons were exchanged against deuterons. The amide I band at 1,645 cm^{-1} was quite symmetric in shape and appeared to be composed of one single major component, with the possibility of a very minor component on the right shoulder. Therefore, no attempt was made to deconvolute this band into

[1]Our D_2O was only 99.8% isotopically pure and some additional H_2O was inevitably introduced into the sample when melittin was added.

components, and the ATR-dichroic ratios were determined directly from the peak heights at 1,645 cm^{-1}. R^{ATR} was 1.39 ± 0.10 for the amide I band of melittin bound to single SPBs of POPC/POPC:POPG (4:1) (Table 3).

In principle, α-helical and unordered residues can contribute to the amide I vibration at almost identical wavenumbers and it is difficult to extract precise proportions of α-helix and random coil from IR spectra. However, the fraction of α-helical residues of melittin in membranes, f_H, has been determined before by CD and Raman spectroscopies to be 0.76 (Vogel and Jahnig, 1986; Vogel, 1987). With this additional information, the order parameter of the helical portion of melittin, S_H, was calculated from the ATR-dichroic ratio of the amide I band by means of Eq. 5. With $\alpha = 39°$ (Tsuboi, 1962; Bradbury et al., 1962), S_H was -0.53 ± 0.17 (Table 3). Since, by definition, order parameters cannot become smaller than −0.5 (or larger than 1.0; see Materials and Methods), the physically acceptable range for S_H is between −0.50 and −0.37. Although a value of $\alpha = 39°$ has been accepted by several researchers (Rothschild et al., 1980; Nabedryk and Breton, 1981; Vogel et al., 1983), α may be as low as 24° (Miyazawa and Blout, 1961; Rothschild and Clark, 1979), in which case the upper limit for S_H becomes -0.29 ± 0.09. The order parameter S_H ($\alpha = 39°$) is close to −0.5 which is the order parameter expected for a (rigid) orientation of the peptide α-helix long axis parallel to the plane of the germanium plate and the supported membrane (i.e., $\theta = 90°$). Both, peptide motions and an average orientation a few degrees away from the plane of the membrane could increase this order parameter. Therefore, even if one allows for some motions of the peptide in the membrane and, perhaps, a slight bend of the α-helix of melittin near proline 14 (Terwilliger and Eisenberg, 1982; Inagaki et al., 1989), the evidence for an average orientation of melittin parallel to the plane of the membrane in SPBs is compelling. The peptide order parameter is in marked contrast to the lipid order parameters in the same sample (Table 2) which are positive, indicating that, in all likelihood, the phospholipids are oriented perpendicular to the plane of the supporting germanium plate (see above). An exchange of D_2O against a buffer of 10 mM Na-phosphate, pD = 7.1, 0.15 M NaCl in D_2O did not significantly change the order parameters (orientations) of the phospholipids (Table 2) or the peptide (Table 3). Because excess unbound melittin is removed by this wash procedure, this experiment provides further evidence that the measured ATR-IR spectra with melittin originate predominantly from membrane-bound melittin. ATR-dichroic ratios were also measured for melittin directly adsorbed to the germanium plate in the absence of the bilayer. The measured values of R^{ATR} and S_H were close to those measured in the presence of the SPB (Table 3). This is not unexpected, if one considers that like quartz, germanium probably also exhibits a negatively charged surface at a neutral pD to which melittin may adsorb horizontally as an amphiphilic α-helix.

These results on the orientation of melittin in SPBs are in marked contrast to those of an earlier polarized ATR-IR study on the orientation of melittin in dry multibilayers (Brauner et al., 1987). To determine whether this discrepancy is caused by the different types

TABLE 3 Frequencies, dichroic ratios, and derived order parameters of the amide I mode for an α-helix

Sample	Bulk phase	n^*	$\nu(cm^{-1})$	R^{ATR}	$S_H^{\ddagger}$
Melittin	D_2O	3		1.38 ± 0.01	$-0.55^{+0.02}_{-0.01}$
Melittin	Air	4	1653 ± 2	1.19 ± 0.03	$-0.08^{+0.11}_{-0.13}$
Supported single planar bilayer (SPBs):					
+POPC//POPC:POPG (4:1)	D_2O	7	1644 ± 2	1.39 ± 0.10	$-0.53^{+0.16}_{-0.17}$
+POPC//POPC:POPG (4:1)	D_2O-Buffer	3	1645	1.42	−0.48
Multibilayers (MBLs):					
+DPPC	Air	7	1653 ± 2	1.28 ± 0.06	$0.25^{+0.18}_{-0.22}$
+POPC	Air	7	1655 ± 1	1.41 ± 0.11	$0.62^{+0.25}_{-0.31}$
+POPC:POPG(4:1)	Air	7	1653 ± 3	1.31 ± 0.16	$0.34^{+0.42}_{-0.60}$

*Number of measurements.
‡Calculated with $\alpha = 39°$ and $f_H = 0.76$.

TABLE 4 **Dichroic ratios and derived order parameters of three characteristic lipid vibrations measured on multibilayers**

Lipid	Melittin	n*	2,918/2,922 cm^{-1}‡		2,850/2,853 cm^{-1}§		1,735 cm^{-1}‖	
			R^{ATR}	S^{L}	R^{ATR}	S_L	R^{ATR}	S_L
DPPC	–	7	1.08 ± 0.05	$0.23^{+0.21}_{-0.18}$	1.03 ± 0.05	$0.44^{+0.26}_{-0.21}$	1.11 ± 0.08	$0.26^{+0.28}_{-0.23}$
DPPC	+	8	1.06 ± 0.05	$0.31^{+0.23}_{-0.19}$	1.02 ± 0.08	$0.49^{+0.47}_{-0.34}$	1.13 ± 0.12	$0.20^{+0.43}_{-0.31}$
POPC	–	7	1.13 ± 0.09	$0.05^{+0.35}_{-0.26}$	1.08 ± 0.09	$0.23^{+0.42}_{-0.30}$	1.10 ± 0.10	$0.29^{+0.38}_{-0.28}$
POPC	+	7	1.17 ± 0.05	$-0.08^{+0.16}_{-0.14}$	1.14 ± 0.07	$0.01^{+0.25}_{-0.20}$	1.17 ± 0.06	$0.09^{+0.17}_{-0.15}$
POPC/POPG (4:1)	–	4	1.08± 0.05	$0.23^{+0.21}_{-0.18}$	1.03 ± 0.05	$0.44^{+0.26}_{-0.21}$	1.03 ± 0.04	$0.54^{+0.17}_{-0.15}$
POPC/POPG (4:1)	+	7	1.11 ± 0.08	$0.11^{+0.33}_{-0.25}$	1.10 ± 0.08	$0.15^{+0.34}_{-0.26}$	1.13 ± 0.14	$0.20^{+0.52}_{-0.35}$

*Number of measurements.
‡Antisymmetric CH_2 stretch (2,918 cm^{-1}: DPPC; 2,922 cm^{-1}: POPC and POPC/POPG).
§Symmetric stretch (2,850 cm^{-1}: DPPC; 2,853 cm^{-1}: POPC and POPC/POPG).
‖Carbonyl stretch.

of model membranes that were used, we repeated some of the experiments by Brauner et al. (1987). In addition, we performed some measurements with MBLs which contained 20 mol% POPG to test whether the presence of negatively charged lipids had any effect on the orientation of the peptide in these membranes. Again, the same three characteristic lipid vibrations were evaluated to determine the order of the phospholipids in the presence and absence of the peptide. In all cases, spectra which are very similar to the ones presented by Brauner et al. (1987) were obtained. The wavenumbers and R^{ATR} values of these measurements are summarized in Table 4. The symmetric and antisymmetric CH_2 stretching modes consistently occured about four wavenumbers lower in MBLs of DPPC than in MBLs of POPC and POPC/POPG (4:1). Although these bilayers are dry, this spectral shift is reminscent of that observed when gel phase and liquid-crystalline lipid bilayers are compared (Cameron et al., 1980). Most of the R^{ATR} values were close to R^{iso} which is the value expected for an isotropic lipid distribution or for lipids oriented close to the magic angle (54.7°). In all cases, except one, however, R^{ATR} was smaller than R^{iso}, which yielded (small) positive lipid order parameters. Again, the average lipid order parameters, S_L, were calculated for each MBL preparation (Table 4). The presence or absence of melittin (10 mol% in the case of DPPC, 3.3 mol% in the cases of POPC and POPC/POPG [4:1]) did not change much the values of R^{ATR} and S_L of the lipids, with the main effect being a wider distribution of R^{ATR} and S_L in a set of 4–8 individual measurements. In general, our values for MBLs of DPPC and POPC, with and without melittin, are in good agreement with those obtained by Brauner et al. (1987). The lipid order parameter which was derived from MBLs of POPC/POPG (4:1) was the highest of all three lipid preparations, but was still comparable with the values from DPPC and POPC. Compared with the lipid order parameters derived from the SPBs (Table 2), the values derived from the MBLs are generally lower. This may indicate either a tilt of the phospholipid director away from the normal on the ATR plate (as suggested by Brauner et al., 1987) or a wider distribution of tilt angles due to imperfect ordering of the phospholipids in MBLs. It is unlikely that the smaller order parameter in MBLs is due to additional motional averaging (as we suggested above for the SPBs), because rapid motional averaging requires the presence of water on the surface of the bilayers.

The order parameter of the helical part of the peptide was determined by measuring the amide I ATR-dichroic ratios from polarized difference spectra (germanium/MBL[+ melittin]/air − germanium/air). In contrast to the difference spectra which were used to determine the order of the peptide in SPBs, these difference spectra also contained the spectral contributions from the lipids.[2] The amide I bands were centered at about 1,654 cm^{-1} and the R^{ATR} values varied from 1.28 to 1.42 in the three different lipid preparations (Table 3). These dichroic ratios are clearly above 1.21 which is expected

[2]Generating difference spectra of the type (germanium/MBL [+melittin]/air-germanium/MBL[-melittin]/air) would not improve the baselines, because it is very difficult to prepare MBLs with equally distributed phospholipids on the germanium plate in two seperate experiments.

for isotropic distribution and give rise to positive helical order parameters, S_H. As for the lipid order parameters, our peptide order parameters are in good qualitative agreement with those found by Brauner et al. (1987). Their values for R^{ATR} were somewhat higher than ours in DPPC (1.46 ± 0.03) and somewhat lower than ours in POPC (1.20). The small numerical discrepancies may be due to the fact that Brauner et al. (1987) used band deconvolution procedures before calculating R^{ATR}, whereas our values of R^{ATR} were measured directly from the raw spectra.

In conclusion, this work shows that independent of the lipid composition, melittin exhibits negative order parameters in SPBs and positive order parameters in MBLs. In contrast, the lipid order parameters are always positive in SPBs and MBLs. The most likely interpretation of these results is that melittin is oriented approximately parallel to the plane of the membrane in hydrated SPBs and approximately parallel to the fatty acyl chains of the lipids in dry MBLs. These results are further consistent with the more general picture that melittin is oriented parallel to the membrane surface in fully hydrated model membranes (Stanislawski and Ruterjans, 1987; Altenbach and Hubbell, 1988; Altenbach et al., 1989) and parallel to the fatty acyl chains in dry or partially hydrated model membranes (Vogel et al., 1983; Vogel, 1987; Brauner et al., 1987). A recent model of the action of melittin on the ionic permeability of lipid bilayers also emphasizes that melittin first binds to membranes in a voltage independent step with its α-helix oriented parallel to the surface of the membrane (Tosteson et al., 1990). Only when a membrane potential (positive on the side of melittin addition) is applied, the *N*-terminus which carries fewer positive charges than the *C*-terminus moves toward the opposite side of the membrane so that the axes of the α-helices become more perpendicular to the surface of the membrane. According to this model, melittin monomers and dimers would then aggregate to form conducting channels with a distribution of aggregation numbers, averaging at four monomers per conducting unit. It may be possible to further test some aspects of this model by ATR-IR spectroscopy on supported planar bilayers. Also, ATR-IR spectroscopy of polypeptides associated with supported planar membranes in D_2O may open new future possibilities for studying the structure and orientation of membrane proteins in a physiological environment.

We thank Dr. U Fringeli, University of Vienna, for lending us his ATR accessory and Dr. J. Wirz, University of Basel, for letting us use his IR spectrometer. We further acknowledge Dr. Fringeli for several helpful discussions.

This work was supported by the Swiss National Foundation grant 31-26849.89.

Received for publication 18 March 1991 and in final form 5 June 1991.

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